Promotion Effect of Sulfite on Deoxyosones and 4-Methylimidazole in Caramel

Model System

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1 **Abstract:** In this study, hydrogen-deuterium (H/D) exchange experiment was carried
2 out to reveal the promotion effect of sulfite on the formation of deoxyosones and
3 4-methylimidazole (4-MeI) in the Maillard reaction. Glucose-ammonium (40 mmol/L,
4 pH 7.4 in PBS) model systems with different levels of sulfite were incubated at
5 110 °C for 2 h. Alpha-dicarbonyls were detected after derivatization by a
6 high-performance liquid chromatography with a diode array detector (HPLC-DAD).
7 4-MeI in the Maillard reaction was tested using a high-performance anion exchange
8 chromatography with an electrochemical detector (HPAEC-ED). The H/D exchange
9 ratios of hexose (fructose, glucose and mannose) were tested by HPAEC-MS. Results
10 showed that the aldo-enol transition of enediol was promoted by sulfite, which
11 promoted the formation of deoxyosones and 4-MeI. In addition, the oxidation reaction
12 of enediols was inhibited by the antioxidant sulfite, which caused the inhibition of
13 osones formation in the Maillard reaction.

14 **Keyword:** 4-methylimidazole, alpha-dicarbonyls, sulfite, enediol, Class IV caramel
15 color, H/D exchange
16

1. Introduction

Caramel color is widely applied for coloring a variety of foods and beverages (Chappel & Howell, 1992). Four classes of caramel color (classes I, II, III, and IV) are divided on the basis of the application and the reactants used in the caramel color manufacture (Hengel & Shibamoto, 2013). Typically, class IV compounds, widely used in soft drinks, are produced thermally by carbohydrates with both sulfite-containing and ammonium-containing compounds. However, many studies on the toxicity of caramel color reported that class IV caramel color contained carcinogenic compounds such as 4-methylimidazole (4-MeI) (Brusick, Jagannath, Galloway, & Nestmann, 1992; Moon & Shibamoto, 2010). Thus, a great deal of attention was attracted among US and European food safety agencies (Lee, Jang, & Shibamoto, 2012).

Except for the addition into the manufacture of caramel color, sulfite is the most widespread anti-brown agent which inhibits the formation of the brown polymer in the final Maillard reaction stage (Y.-G. Guan, Zhu, Yu, Xu, & Zhu, 2013; Iyengar & McEvily, 1992). Meanwhile, the addition of sulfite in the Maillard reaction is also used to control harmful compounds formation, including 4-MeI (Jang, Jiang, Hengel, & Shibamoto, 2013), 5-hydroxymethyl-2-furaldehyde (Y. G. Guan, Zhu, Yu, Xu, & Shi, 2011) and N-epsilon-(carboxymethyl)-lysine (X.-B. Xu, Ma, Yu, & Guan, 2013). Jang et al. (Jang, Jiang, Hengel, & Shibamoto, 2013) also reported that adding sulfite was effective on inhibiting dicarbonyls formation such as methylglyoxal (MGO). Noted that sulfite based on the reports mentioned above always shows inhibitory

behavior in control of the Maillard reaction due to the nucleophilic property of sulfite, which is facile to adduct with carbonyl group from carbohydrates, Amadori rearrangement products (ARPs) or dicarbonyls and subsequently blocks the further reaction of carbonyls to form the relevant compounds. Recently, Lee et al. (Lee, Jang, & Shibamoto, 2012) reported the promotion effect of sulfite on 4-MeI formation, but the detailed explanation was not proposed. Therefore, a profound mechanism research should be carried out to comprehensively understand the role of sulfite in the Maillard reaction.

MGO, a proposed precursor compound of 4-MeI, has been detailed on its formation via carbohydrate degradation undergoing caramelization and the Maillard reaction (Weigel, Opitz, & Henle, 2004). The origin of MGO from reported views has included sugars, ARPs and long-chain dicarbonyls (such as 3-deoxyglucosone (3-DG) and 1-deoxyglucosone) regardless of Schiff base (Davidek, Clety, Aubin, & Blank, 2002; Gobert & Glomb, 2009; Usui, Yanagisawa, Ohguchi, Yoshino, Kawabata, Kishimoto, et al., 2007). Subsequently, Gobert et al. (Gobert & Glomb, 2009) summarized that three major pathways leading to the formation of MGO were retro-aldol reactions, α - and β -dicarbonyl cleavages. In addition, during heating and storage, MGO formed from the Maillard reaction is recognized as an important precursor of 4-MeI (Moon & Shibamoto, 2010). Thus, it is important to control the formation of MGO in the Maillard reaction.

In this study, the H/D exchange experiment was carried out to investigate the mechanism of sulfite on the promotion of 4-MeI and deoxyosones in the Maillard

reaction. In addition, the precursor compounds, such as glucosone, 3-DG and MGO, were also monitored for proposing the appropriate mechanism for effect of sulfite on the 4-MeI formation.

2. Materials and Methods

2.1 Chemicals

Methanol was chromatographic grade and purchased from Merck (Damstadt, Germany). D₂O (99%), D-glucose (Glc, 99%), arabinose (99%), ammonium hydroxide solution (25%), o-phenylenediamine (OPD, 99%), sodium sulfite (98%), disodium hydrogen phosphate dodecahydrate (98%) and sodium dihydrogen phosphate dehydrate (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (50%) used for HPAEC analysis was chromatographic grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Preparation of class III and IV caramel color

Different levels of sodium sulfite (the final concentration of sulfite: 0, 20, 40, 100 or 400 mmol/L) were spiked into the solution of Glc-NH₄⁺ (40 mmol/L, 1:1) in PBS (0.2 mol/L, pH 7.4). Four milliliter mixtures were incubated at 110 °C for up to 120 min in a swing-top bottle. After a defined reaction time, the reaction mixtures were cooled to room temperature by iced water immediately to stop the reaction. One milliliter reaction mixture was incubated using OPD (1 mL, 0.5 mg/mL in 0.2 mol/L PBS of pH 7.4) for 12 h at room temperature and the rest of reaction mixture was stored at 4°C for further analyses.

2.3 H/D exchange samples in the Maillard reaction

According to Biemel et al. (Biemel, Conrad, & Lederer, 2002), D₂O was used as the deuterium source of the Maillard reaction, Glc-NH₄⁺ (40 mmol/L, 1:1) in phosphate buffer deuterioxide solutions (0.2 mol/L, pH 7.4) was reacted at 110 °C for up to 120 min after adding different levels of sulfite sodium (the final concentration of sulfite: 0, 20 and 40 mmol/L, respectively.). After a defined reaction time, the reaction mixtures were cooled to room temperature by iced water immediately to stop the reaction.

2.4 Syntheses of Quinoxalines

Quinoxalines were prepared according to the method of Morita et al. (1983) with a slight modification (Morita, Mizutani, Hayashi, Kirihata, Ichimoto, Ueda, et al., 1983). Glucose or arabinose was mixed with OPD and stirred for 4 h at 60 °C in 20 mL aqueous solution. The crude products were purified by a C₁₈ (10 µm) column (30 × 200 mm, inside diameter × length, lisui II) with a ultraviolet detector (Lisui, Suzhou Lisui Technology Co., Ltd, China) by elution with methanol /water (2:8, v/v) at a flow rate of 18 mL/min. The isolated quinoxalines was collected and dried in vacuum. The solvent was eliminated by vacuum concentration, and then freeze-dried (white powder, 60 mg). ¹H NMR spectra was recorded on a Bruker AVANCE AV 400 spectrometer with DMSO as solvent at 400 MHz at 30 °C. HRMS was worked on a Bruker maXis impact mass spectrometer instrument.

Glucosone-Quinoxaline (Q, t_R 15-17 min). HR-MS: m/z 273.0852 (found); m/z 273.0846 (calculated for C₁₂H₁₄N₂O₄Na) [M+Na]⁺. ¹H NMR (400 MHz, in DMSO-d₆): δ/ppm=3.34 (s, 4H), 3.66 (dd, J=4 Hz, 1H), 4.65 (m, 1H), 5.14 (d, J=2 Hz, 1H), 5.59 (d, J=1.2 Hz, 1H), 7.80 (m, 2H), 8.05 (m, 2H), 9.08 (s, 1H).

Pentosone-Q (t_R 16.5-17.3 min). HR-MS: verified m/z 243.0743 (found); m/z 243.0740 (calculated for $C_{11}H_{12}N_2O_3Na$) $[M+Na]^+$. 1H NMR (400 MHz, in DMSO- d_6): $\delta/ppm=3.47$ (m, 1H), 3.61 (m, 1H), 3.90 (m, 1H), 4.78 (d, $J=4$ Hz, 1H), 7.76-7.84 (m, 2H), 8.01-8.07 (m, 2H), 9.05 (s, 1H).

Threosone-Q (t_R 17.5-18.5 min). HR-MS: verified m/z 213.0635 (found); m/z 213.0634 (calculated for $C_{10}H_{10}N_2O_2Na$) $[M+Na]^+$. NMR results were in line with Usui et al. (Usui, et al., 2007).

3-Deoxyglucosone-Q (3-DG-Q, t_R 18-20 min). HR-MS: m/z 257.0896 (found); m/z 257.0897 (calculated for $C_{12}H_{14}N_2O_3Na$) $[M+Na]^+$. 1H NMR (400 MHz, in $CDCl_3$): $\delta/ppm= 3.14$ (q, $^2J= 14.1$ Hz, $^3J= 9.7$ Hz, 1H), 3.43 (q, $^2J=14.0$ Hz, $^3J=2.8$ Hz, 1H), 3.61 (m, 1H), 3.65 (q, $^2J=11.4$ Hz, $^3J=5.7$ Hz, 1H), 3.80 (q, $^2J=11.0$ Hz, $^3J=3.7$ Hz, 1H), 4.09 (m, 1H), 7.80 (m, 2H), 8.05 (m, 2H), 8.85 (s, 1H).

Hydroxypyruvaldehyde-Q (HPA-Q, t_R 20-23 min). 1H NMR (400 MHz, in DMSO- d_6): $\delta/ppm=4.83$ [s, 2H, $-CH_2(OH)$], 7.76-7.84 (m, 2H), 8.01-8.07 (m, 2H), 9.05 (s, 1H, $N=CH-$); HR-MS: m/z 161.0709 (found); m/z 161.0712 (calculated for $C_9H_9N_2O$) $[M+H]^+$.

MGO-Q was verified in comparison to commercial available materials.

2.5 Analyses of dicarbonyls by HPLC-UV

Identification of alpha-dicarbonyls as quinoxalines was carried out according to Gobert and Glomb (Gobert & Glomb, 2009) with slight modifications. The analyses were performed in an HPLC-DAD system, which consisted of a 5 μm Waters Atlantis T3 150 $\times$ 4.6 mm column (Waters Co., USA), a Waters 600 pump and a Waters 2998

diode array detector (Waters Co., USA). 1.0 mL reaction solution was mixed with 1 mL OPD solution (0.5 mg/mL), and reacted at 25°C for 12 h in dark. The injection volume was 20 µL (filtered through 0.45 µm filters), and the mobile phases were methanol and water, and set as 5 %v/v methanol, 0-5 min, then a linear gradient from 5 %v/v methanol to 30 %v/v methanol, 5-10 min, subsequently, changed to 40 %v/v methanol, 10-45 min, at last, 100 %v/v methanol was used for elution from 45 to 65 min at a flow rate of 1 mL/min. Column temperature was set at 25 °C. Spectral data from all peaks were accumulated in the range of 200-600 nm, and chromatograms were recorded at 315 nm.

2.6 Analyses of 4-MeI by HPAEC-ED

The analysis of 4-MeI was carried out according to the literature (X. B. Xu, Liu, Yu, Yu, & Zhao, 2015), Ansys SPEC SCX Disc 15 mg/3 mL cartridge (Varian, Walnut Creek, CA) was conditioned with 1 mL methanol and 1 mL water. The 3 mL Maillard reaction mixtures acidified with 20 µL HCl (0.1 mol/L) were loaded and passed through the cartridge. The retained 4-MeI was eluted out with 6 mL methanol/ammonia (5%, v/v). The collected extract was evaporated by vacuum concentration at 39 °C, and the residues were dissolved by 3 mL deionized water for HPAEC-ED analyses.

The pretreated samples were filtered through a Millex-HN nylon clarification kit of 0.45 µm pore size (Millipore, Bedford, MA), and then analyzed on a DX 5000 Dionex system (Dionex Corp., Sunnyvale, CA), which consisted of a gradient pump (model EG40) with on-line degassing and an electrochemical detector (ED, ED40, Dionex

Corp., Sunnyvale, CA). Separation was accomplished on an amino trap column (50×4 mm, Dionex, P/N: 046122). The mobile phase was isocratic elution with 100 mM NaOH at a flow rate of 0.5 mL/min. The injection volume was 20 µL. The pH reference electrode and gold electrode were used in HPAEC-ED analyses. The waveforms were applied under the following settings: $E_1=0.13$ V ($t_1=0.04$ s), $E_2=0.33$ V ($t_2=0.16$ s), $E_3=0.55$ V ($t_3=0.24$ s), $E_4=0.33$ V ($t_4=0.09$ s), $E_5=-1.67$ V ($t_5=0.01$ s), $E_6=0.93$ V ($t_6=0.01$ s), $E_7=0.13$ V ($t_7=0.01$ s). Integration occurred from 0.20 to 0.44 s during E_3 application.

The regression equation for 4-MeI standard was as follows: $y_1=16.480x_1+0.479$; $R^2=0.9999$, where x_1 was 4-MeI concentration, mg/L; y_1 was the peak area of 4-MeI. The retention time of 4-MeI was about 8.5 min, and the detection range was 0.100 to 10.000 mg/L in this test, the recovery of the 4-MeI in reaction mixtures was about 96 % (w/w).

2.7 Glucose and its tautomer analyzed by HPAEC-MS

According to the literature (X. B. Xu, Liu, Guo, Yu, & Yu, 2014), H/D exchange ratio of glucose and its tautomers (fructose and mannose) in the Maillard reaction was analyzed by HPAEC-MS. A Dionex ICS 5000 system (Dionex Corp., USA) coupled with a gradient pump (model EG40) with on-line degassing was used for HPAEC. The separation was performed on a CarboPac PA1 (2 mm×250 mm) column with a CarboPac PA1 guard (2 mm×50 mm) column (Dionex). A flow rate of 0.30 mL/min at 30 °C was used for separations. A two-step gradient was used as follows: 10 mmol/L NaOH (0-25 min) for monosaccharide analysis, and 100 mmol/L NaOH (25-45 min)

for disaccharide analysis. The injection volume was 25 μ L. For conversion of the eluate into an ESI-compatible solution, an ASRS 300 suppressor (2 mm; Dionex, USA) was used as an in-line desalter; it was set at a constant current of 75 mA and operated in external water mode (regenerant flow rate approximately 3 mL/min). The converted chromatographic effluent was introduced into the ESI-MS for sugar analysis. Xcalibur software was used for auto-control of the entire system and data acquisition.

The ESI-MS operating conditions were as follows: electrospray voltage 5.0 kV, nitrogen sheath gas flow 9 L/min, and nitrogen auxiliary gas flow 3 L/min. The temperature of the heated capillary was set at 275 °C. A sheath liquid consisting of 50 mM NH_4Ac in isopropanol with 0.05% acetic acid, infused into ESI source at a flow rate of 3 μ L/min. The MS of glucose, fructose and mannose was detected as quasi-molecular ions $[\text{M}+\text{NH}_4]^+$ in positive-ion mode, the value of mass-to-charge ratio (m/z) was 198, the deuterated (d) sugars in the Maillard reaction contained hexose-d and hexose-d₂, m/z value of which was 199 and 200, respectively.

The regression equation for glucose standard was as follows: $y_2=52.263x_2-821.6$; $R^2=0.9993$, where x_2 was glucose concentration, mg/L; y_2 was the peak area of glucose. The regression equation for fructose standard was as follows: $y_3=40.235x_3-741$; $R^2=0.9869$, where x_3 was fructose concentration, mg/L; y_3 was the peak area of fructose. The regression equation for mannose standard was as follows: $y_4=65.124x_4-596$; $R^2=0.9989$, where x_4 was mannose concentration, mg/L; y_4 was the peak area of mannose.

Percent label distribution of deuterated hexose was calculated as follows: $W_{m/z}^a (\%) = M_{m/z}^a / (M [\text{hexose}] + M [\text{hexose-d}] + M [\text{hexose-d2}]) \times 100$. Where $W_{m/z}^a$ was the percent label distribution of sugar a with the value of m/z, $M_{m/z}^a$ was the weight of sugar a with the value of m/z, M [hexose] was the weight of hexose (m/z=198), M [hexose-d] was the weight of hexose-d (m/z=199), M [hexose-d2] was the weight of hexose-d2 (m/z=200).

2.8 Statistical analysis

Means and standard deviations of the data were calculated for each treatment. Analysis of variance (ANOVA) was carried out to determine any significant differences ($p < 0.05$) among the applied treatments by the SPSS software package (SPSS 10.0 for windows).

3 Results and discussion

3.1 Identification of alpha-dicarbonyl as quinoxalines with HPLC-UV

Briefly, six main dicarbonyls, i.e. glucosone, pentosone, threosone, 3-DG, HPA and MGO were detected using HPLC-UV methods (Fig. 1). The C₁₈-column showed excellent resolution for all dicarbonyls separation under the optimized HPLC condition, and all target compounds were eluted out before 30 min.

3.2 Effect of sulfite on the formation of dicarbonyls

In the present study, the addition of sulfite significantly promoted the formation of deoxyosones (MGO and 3-DG) (Fig. 2A and 2B). After 15 min heating, the formed levels of MGO were up to 0.1, 11.2, 16.9, 28.1 and 37.1 mmol/L in the model systems with 0, 20, 40, 100 or 400 mmol/L of sulfite addition, respectively. Meanwhile, the

formed levels of 3-DG were up to 0.63, 0.75, 1.37, 1.78 and 2.61 mmol/L in the model systems in the presence of 0, 20, 40, 100 or 400 mmol/L of sulfite, respectively. Moreover, as the heating time was increasing, the levels of deoxyosones in all model systems with sulfite were higher than that in the model system without sulfite.

Besides, the addition of sulfite significantly inhibited the formation of osones (HPA, pentosone, threosone and glucosone) in model reaction systems. The changes of osones were shown in Fig. 2C, 2D, 2E and 2F. HPA and threosone were two major osones in model system without sulfite. After 15 min heating, the contents of HPA and glucosone in model systems without sulfite were increased to the highest concentration (i.e. 7.1 and 0.79 mmol/L, respectively) and then decreased along with the increase of heating time. And the highest contents of pentosone and threosone (i.e. 0.31 and 2.4 mmol/L, respectively) were tested after 30 min heating. In addition, the levels of osones in all model systems with sulfite were lower than that in the model system without sulfite and the contents of all the four osones were decreased with the increasing sulfite levels, even nearly no HPA, pentosone, threosone and glucosone were detected in the 90 min reaction after adding 400 mmol/L sulfite.

Sulfite as a nucleophile generally attacked the carbon atom of the carbonyls to form the hydroxysulphonate. Wedzicha et al. (Wedzicha, Lamikanra, Herrera, & Panahi, 1984) proposed that sulfite ion could reacted with dicarbonyls (such as 3-DG, 3,4-dideoxyhexosulos-3-ene) to form less reactive products like 3,4-dideoxy-4-sulphohexosulose, which blocked the formation of color. Therefore, sulfite was generally applied to inhibit the non-enzymic browning (Wedzicha &

Kaban, 1986). In addition, Jang et al. (Jang, Jiang, Hengel, & Shibamoto, 2013) proposed that sulfite formed adducts with aldehyde which contributed to the inhibition of 4-MeI formation. Thus, it was concluded that the level of carbonyls in the Maillard reaction would be reduced after sulfite addition due to the formation of adducts of carbonyls with sulfite. In this study, the decrease of osones (such as glucosone, pentosone, HPA and so on) after the addition of sulfite could be explained according to these previous reports. However, the proposed mechanism cannot totally explain the change of deoxyosones (such as 3-DG and MGO) as the addition of sulfite significantly promoted the formation of MGO and 3-DG (Fig. 2A and 2B). The result excluded the fact that the formation of sulfite-aldehyde adducts caused the reduction of dicarbonyls. Therefore, further experiments were required to study the promotion mechanism of sulfite on deoxyosones formation.

3.3 H/D exchange and tautomerization of glucose in the Maillard reaction

Further insights to the precise enolization pathway from glucose in the Maillard reaction were achieved by H/D exchange experiment. Based on the report in recent literatures, osones were originated from enediol via oxidative reaction (Gobert & Glomb, 2009; Voigt, Smuda, Pfahler, & Glomb, 2010). For example, glucosone was produced by glucose oxidation degradation (Usui, et al., 2007). Pentosone, the direct oxidation product of enediol intermediate was detected in aerated glucosone incubations by Gobert and Glomb (Gobert & Glomb, 2009). In addition, threosone was preferentially formed under aerated conditions in incubation with glucose (Usui, et al., 2007). HPA was formed by the oxidation of glyceraldehyde, which was

generated from glucose, 3-DG or glucosone via the retro-aldol reaction (Usui, et al., 2007). Therefore, the inhibition of osones formation in this study suggested that the suppression of oxidation of enediol in the Maillard reaction system or decrease of enediol levels was caused by sulfite. In H/D exchange experiment, the total percent distribution of deuterated hexoses was increased from 77.3% (0 mmol/L sulfite) to 100% (40 mmol/L sulfite) after sulfite addition (Table 1.). The result indicated that sulfite promoted the eneolization of glucose, which caused the increase of enediols levels. Therefore, the inhibition of the osones formation was attributed to the suppression of oxidation of enediol. Moreover, the antioxidation of sulfite had been reported in the literature. Lancia et al.(Lancia, Musmarra, Pepe, & Prisciandaro, 1997) indicated that the dissolved oxygen in solution could be absorbed via sulfite oxidation. During the oxidation process, sulfite ion was converted into sulfate ion. Based on the mechanism of sulfite oxidation, sulfite ion in the Maillard reaction system had an ability to exhaust dissolved oxygen in solution and subsequently inhibited the oxidation of enediol to form osones.

Though the formation of osones was inhibited in the Maillard reaction with the addition of sulfite, the formation of deoxyosones (3-DG and MGO) was significantly promoted by sulfite in this research. Early researchers had established the formation pathways of 3-DG and MGO in the Maillard reaction or caramelization. Gobert and Glomb (Gobert & Glomb, 2009) indicated oxidative mechanisms of MGO formation were absent. In addition, 3-DG was confirmed as the precursor of MGO in recent reports (Hollnagel & Kroh, 1998; Thornalley, Langborg, & Minhas, 1999; Weenen,

1998; Yaylayan & Keyhani, 2000). Weenen (Weenen, 1998) suggested that the formation of MGO was mainly degraded from 3-DG via the retroaldolisation, which was further confirmed by Yaylayan and Keyhani (Yaylayan & Keyhani, 2000). Besides, the formation of 3-DG under non-oxidative condition was also confirmed by Usui et al.(Usui, et al., 2007). Therefore, the oxidation pathway was not required for the formation of deoxyosones.

In this study, the novel evidence that sulfite promoted the aldo-enol transition of 1,2-enediol in the Maillard reaction was achieved from the percent distribution of deuterium tautomerism sugar in H/D experiment. The detailed mechanism of H/D exchange was shown in Fig. 4. When glucose-NH₄⁺ system did not contain sulfite, the percent distribution of deuterated hexose was listed in table 1. Glucose of 21.4% (m/z=198), fructose-d of 24.4% (m/z=199) and fructose-d₂ of 24.6% (m/z=200) were mainly detected in model reaction system, whereas the deuterated/undeuterated mannose and deuterated glucose were tested at a relatively low proportion. Therefore, when sulfite was not added into model reaction system, the isomerization of D-glucose into D-fructose was primarily observed. According to the Lobry de Bruyn–Alberda van Ekenstein transformation (De Wit, Kieboom, & van Bekkum, 1979), glucose (1) was firstly transformed into transition state anion compound 12, when the deuterium ion attacked the oxygen atom at the C1 or C2 hydroxyl group with the same chance, the compound 12 was equally transformed via 1,2-eneolization into enediol 3 or 11, keto-enol transition of which was converted into deuterated fructose, including fructose-d (24.4%, m/z=199) and fructose-d₂ (24.6%, m/z=200).

However, when sulfite (40 mmol/L etc.) was spiked into the model reaction system, the proportion of deuterated fructose was significantly decreased (4.8% for fructose-d, 12.8% fructose-d₂), whereas the proportion of deuterated glucose was increased from 13.0% to 30.7% (glucose-d), and from 4.1% to 14.4% (glucose-d₂), and the proportion of deuterated mannose was increased from 8.9% to 27.1% (mannose-d), and from 2.3% to 10.3% (mannose -d₂). The result indicated that sulfite promoted the aldo-enol transformation of enediol compound 3 into glucose and mannose rather than the keto-enol transformation into fructose. Because the steric hindrance contributed to sulfite attacking the proton of hydroxyl at C1 position rather than C2 position, aldo-enol transition of enediol compound 3 was the dominant reaction rather than keto-enol transition of enediol compound 3. It was reported that the retro-enolization via aldo-enol transition was the trigger of beta-C3-elimination reaction of 1,2-enediol leading to 3-DG formation (Anet, 1960). Therefore, the promotion of aldo-enol transition by sulfite accelerated the C3-elimination reaction of 1,2-enediol to form 3-DG, which was subsequently degraded into the increased MGO.

3.4 Effect of sulfite on 4-MeI formation

Fig. 3 showed the changes of 4-MeI formation in the Maillard reaction with and without sulfite. Obviously, when sulfite was added into the Maillard reaction system, a higher concentration of 4-MeI was detected comparing with no sulfite addition. After 15, 30, 60, 90 and 120 min reaction, the levels of 4-MeI ranged from 0.61 to 1.42 mg/L with 100 mmol/L sulfite addition, comparing with 0.07-0.18 mg/L in the reaction system without sulfite addition. When the concentration of sulfite increased

to 200 mmol/L, the 4-MeI levels were reduced, but still higher than that in the model reaction system without sulfite.

Some authors had indicated that MGO was the precursor of 4-MeI formation (Hengel & Shibamoto, 2013). As a matter of course, the accelerated formation of MGO by sulfite definitely contributed to promoting the formation of 4-MeI. Though the levels of 4-MeI were decreased with high level addition of sulfites (>200 mmol/L), the amount of 4-MeI was still higher than that in model reaction without sulfite. Therefore, sulfite did not always show inhibitory behavior in control of the Maillard reaction.

4. Conclusions

In summary, sulfite promoted the aldo-enol instead of keto-enol transition of enediol transformed from hexose, which caused the increase of deoxyosones and 4-MeI. In addition, the antioxidant sulfite inhibited the oxidation of enediol to generate osones. The results in the present study suggested that sulfite played an important role in the caramel preparations and did not always show inhibitory effect on 4-MeI formation during caramel color IV manufacture.

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Reference

- Anet, E. F. L. J. (1960). 3-Deoxyhexosones. *Journal Of The American Chemical Society*, 82(6), 1502-1502.
- Biemel, K. M., Conrad, J., & Lederer, M. O. (2002). Unexpected Carbonyl Mobility in Aminoketoses: The Key to Major Maillard Crosslinks. *Angewandte Chemie International Edition*, 41(5), 801-804.
- Brusick, D. J., Jagannath, D. R., Galloway, S. M., & Nestmann, E. R. (1992). Genotoxicity Hazard Assessment of Caramel Colours Iii and Iv. *Food And Chemical Toxicology*, 30(5), 403-410.
- Chappel, C. I., & Howell, J. C. (1992). Caramel Colours—a Historical Introduction. *Food And Chemical Toxicology*, 30(5), 351-357.
- Davidek, T., Clety, N., Aubin, S., & Blank, I. (2002). Degradation of the Amadori Compound N-(1-Deoxy-D-Fructos-1-Yl)Glycine in Aqueous Model Systems. *Journal Of Agricultural And Food Chemistry*, 50(19), 5472-5479.
- De Wit, G., Kieboom, A. P. G., & van Bakkum, H. (1979). Enolisation and Isomerisation of Monosaccharides in Aqueous, Alkaline Solution. *Carbohydrate Research*, 74(1), 157-175.
- Gobert, J., & Glomb, M. A. (2009). Degradation of Glucose: Reinvestigation of Reactive A-Dicarbonyl Compounds†. *Journal of Agricultural and Food Chemistry*, 57(18), 8591-8597.
- Guan, Y.-G., Zhu, S.-M., Yu, S.-J., Xu, X.-B., & Zhu, L.-C. (2013). < I> Short Communication:</I> Possible Mechanism for Inhibiting the Formation of

369 Polymers Originated from 5-Hydroxymethyl-2-Furaldehyde by Sulfite Groups in
 370 the Dairy Thermal Process. *Journal Of Dairy Science*, 96(5), 2826-2831.

371 Guan, Y. G., Zhu, S. M., Yu, S. J., Xu, X. B., & Shi, W. H. (2011). SO_3^{2-} Effects the
 372 5- Hydroxymethyl- 2- Furaldehyde Content in Ammonium Sulphite–Glucose
 373 Solutions. *International Journal of Food Science & Technology*, 46(5),
 374 1007-1013.

375 Hengel, M., & Shibamoto, T. (2013). Carcinogenic 4(5)-Methylimidazole Found in
 376 Beverages, Sauces, and Caramel Colors: Chemical Properties, Analysis, and
 377 Biological Activities. *Journal Of Agricultural And Food Chemistry*, 61(4),
 378 780-789.

379 Hollnagel, A., & Kroh, L. W. (1998). Formation of Alpha-Dicarbonyl Fragments
 380 from Mono- and Disaccharides under Caramelization and Maillard Reaction
 381 Conditions. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food*
 382 *Research and Technology*, 207(1), 50-54.

383 Iyengar, R., & McEvily, A. J. (1992). Anti-Browning Agents: Alternatives to the Use
 384 of Sulfites in Foods. *Trends In Food Science & Technology*, 3(1), 60-64.

385 Jang, H. W., Jiang, Y., Hengel, M., & Shibamoto, T. (2013). Formation of
 386 4(5)-Methylimidazole and Its Precursors, A-Dicarbonyl Compounds in Maillard
 387 Model Systems. *Journal Of Agricultural And Food Chemistry*, 61(28),
 388 6865-6872.

389 Lancia, A., Musmarra, D., Pepe, F., & Prisciandaro, M. (1997). Model of Oxygen
 390 Absorption into Calcium Sulfite Solutions. *Chemical Engineering Journal*, 66(2),

391 123-129.

392 Lee, K.-G., Jang, H., & Shibamoto, T. (2012). Formation of Carcinogenic
 393 4(5)-Methylimidazole in Caramel Model Systems: A Role of Sulfite. *Food*
 394 *Chemistry*, 136(3), 1165-1168.

395 Moon, J.-K., & Shibamoto, T. (2010). Formation of Carcinogenic
 396 4(5)-Methylimidazole in Maillard Reaction Systems. *Journal Of Agricultural*
 397 *And Food Chemistry*, 59(2), 615-618.

398 Morita, N., Mizutani, M., Hayashi, K., Kiriata, M., Ichimoto, I., Ueda, H., & Takagi,
 399 M. (1983). Quinoxalines Derived from D-Glucose and Maltose with
 400 O-Phenylene-Diamine under Refluxed Conditions in Alkaline Media.
 401 *Agriculture and Biology*, 35, 59-70.

402 Thornalley, P. J., Langborg, A., & Minhas, H. S. (1999). Formation of Glyoxal,
 403 Methylglyoxal and 3-Deoxyglucosone in the Glycation of Proteins by Glucose.
 404 *Biochemical Journal*, 344(1), 109-116.

405 Usui, T., Yanagisawa, S., Ohguchi, M., Yoshino, M., Kawabata, R., Kishimoto, J.,
 406 Arai, Y., Aida, K., Watanabe, H., & Hayase, F. (2007). Identification and
 407 Determination of Alpha-Dicarbonyl Compounds Formed in the Degradation of
 408 Sugars. *Bioscience Biotechnology And Biochemistry*, 71(10), 2465-2472.

409 Voigt, M., Smuda, M., Pfahler, C., & Glomb, M. A. (2010). Oxygen-Dependent
 410 Fragmentation Reactions During the Degradation of
 411 1-Deoxy-D-Erythro-Hexo-2,3-Diulose. *Journal Of Agricultural And Food*
 412 *Chemistry*, 58(9), 5685-5691.

413 Wedzicha, B. L., & Kaban, J. (1986). Kinetics of the Reaction between
 414 3-Deoxyhexosulose and Sulphur(Iv) Oxospecies in the Presence of Glycine.
 415 *Food Chemistry*, 22(3), 209-223.

416 Wedzicha, B. L., Lamikanra, O., Herrera, J. C., & Panahi, S. (1984). Recent
 417 Developments in the Understanding of the Chemistry of Sulphur(Iv) Oxospecies
 418 in Dehydrated Vegetables. *Food Chemistry*, 15(2), 141-155.

419 Weenen, H. (1998). Reactive Intermediates and Carbohydrate Fragmentation in
 420 Maillard Chemistry. *Food Chemistry*, 62(4), 393-401.

421 Weigel, K., Opitz, T., & Henle, T. (2004). Studies on the Occurrence and Formation
 422 of 1,2-Dicarbonyls in Honey. *European Food Research And Technology*, 218(2),
 423 147-151.

424 Xu, X.-B., Ma, F., Yu, S.-J., & Guan, Y.-G. (2013). Simultaneous Analysis of
 425 Nε-(Carboxymethyl) Lysine, Reducing Sugars, and Lysine During the Dairy
 426 Thermal Process. *Journal Of Dairy Science*, 96(9), 5487-5493.

427 Xu, X. B., Liu, D. B., Guo, X. M., Yu, S. J., & Yu, P. (2014). Improvement of Sugar
 428 Analysis Sensitivity Using Anion-Exchange Chromatography-Electrospray
 429 Ionization Mass Spectrometry with Sheath Liquid Interface. *Journal Of*
 430 *Chromatography A*, 1366, 65-72.

431 Xu, X. B., Liu, D. B., Yu, S. J., Yu, P., & Zhao, Z. G. (2015). Separation and
 432 Determination of 4-Methylimidazole, 2-Methylimidazole and
 433 5-Hydroxymethylfurfural in Beverages by Amino Trap Column Coupled with
 434 Pulsed Amperometric Detection. *Food Chemistry*, 169, 224-229.

435 Yaylayan, V. A., & Keyhani, A. (2000). Origin of Carbohydrate Degradation
436 Products in L-Alanine/D-[¹³C] Glucose Model Systems. In *Journal Of*
437 *Agricultural And Food Chemistry*, vol. 48 (pp. 2415-2419).

438 Figure Legends:

439 Figure 1 HPLC chromatograms of dicarbonyl derivatives obtained from the
440 glucose-ammonium solution with or without sulfite.

441 Figure 2 changes of the dicarbonyls as the function of heating time. A, B, C, D, E and
442 F were the levels of 3-deoxyglucoseone (3-DG), methylglyoxal (MGO),
443 hydroxypyruvaldehyde (HPA), pentosone, glucosone and threosone as the function of
444 heating time, respectively.

445 Figure 3 Levels of 4-MeI as the function of sulfite concentration.

446 Figure 4 Proposed chemical mechanism of enediol transition affected by sulfite.

447

Table 1 Percent Deuterium Label Distribution Identified in Glucose/ammonium/sulfite Incubations by ESI-HPAEC/MS

hexose	sulfite (mmol/L)	H/D exchange ratio (w/w , %)		
		m/z=198	m/z=199	m/z=200
glucose	0	21.4	13.0	4.1
	20	3.2	25.4	13.2
	40	Nd.	30.7	14.4
mannose	0	Nd.	8.9	2.3
	20	Nd.	18.9	7.8
	40	Nd.	27.1	10.3
fructose	0	1.3	24.4	24.6
	20	1.2	9.5	20.7
	40	Nd.	4.8	12.8

448 Nd. is not detected.

Figure 1

Figure 2

Figure 3

Figure 4